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JC930 U.S. PTO 09/15/00

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No,	960296.96650
First Inventor or Application Identifier	Judith E. Kimble
Title	ASSAYS FOR MODULATORS OF PROLYL-4 HYDROXYLASE
Express Mail Label No,	EJ636887080US

JC564 U.S. PTO 09/663805 09/15/00

APPLICATION ELEMENTS

See MPEP Chapter 600 concerning utility patent application contents

ADDRESS TO: Assistant Commissioner for Patents
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Washington, D.C. 20231

1 [X] Fee transmittal Form
(Submit an original and a duplicate for fee processing)

2 [X] Specification [Total Pages 18]
(preferred arrangement set forth below)
- Descriptive title of the invention
- Cross References to Related Applications
- Statement Regarding Fed Sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings *(if filed)*
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3 [X] Drawing(s) (35 USC 113) [Total Sheets 3]

4. Oath or Declaration [Total Pages 3]
a. [ ] Newly executed (original or copy)
b. [ ] Copy from prior Application (37 CFR 1.63(d)) (for continuation/divisional with Box 17 completed)
[Note Box 5 below]
i. [ ] DELETION OF INVENTOR(S) Signed Statement attached deleting inventor(s) named in prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

5 [ ] Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference herein.

6 [ ] Microfiche Computer Program *(Appendix)*

7. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
a. [ ] Computer readable Copy
b. [ ] Paper Copy (identical to computer copy)
c. [ ] Statement Verifying identity of above

ACCOMPANYING APPLICATION PARTS

8 [ ] Assignment Papers (cover sheet & documents)
9 [ ] 37 CFR 3 73(b) Statement [ ] Power of Attorney (where there is an assignee)
10 [ ] English Translation Document *(if applicable)*
11 [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
12 [ ] Preliminary Amendment
13 [X] Return receipt postcard (MPEP 503) *(Should be specifically itemized)*
14 [ ] *Small Entity Statement(s) [ ] Statement filed in prior application Status still proper and desired
15 [ ] Certified copy of priority Document(s) *(if foreign priority is claimed)*
16 [ ] Other:

* A newstatement is required to pay small entity fees, except where one has been filed in a prior application and is being relied upon

17. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*

[ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application no. ___/_____

Prior application information: Examiner: Group/Art Unit:

18. CORRESPONDENCE ADDRESS

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Signature	Jean C. Baker	Date	September 15, 2000

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FEE TRANSMITTAL for FY 2000

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Small Entity payments must be supported by a small entity statement
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See 37 C.F.R. §§1.27 and 1.28

TOTAL AMOUNT OF PAYMENT	$708.00

Complete if Known	
Application Number	
Filing Date	September 15, 2000
First Named Inventor	Judith E. Kimble
Group Art Unit	
Examiner Name	
Attorney Docket Number	960296.96650

METHOD OF PAYMENT (check one)

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Deposit Account Name: Quarles & Brady LLP

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2. [ ] Payment Enclosed:

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	690	201	345	Utility filing fee	$690.00
106	310	206	155	Design filing fee	
107	480	207	240	Plant filing fee	
108	690	208	345	Reissue filing fee	
114	150	214	75	Provisional filing fee	

SUBTOTAL (1) ($)690.00

2. CLAIMS

			Extra		Fee from below		Fee Paid
Total Claims	21	-20**=	1	X	18 00	=	18 00
Independent	3	-3**=	0	X	0	=	0
Multiple Dependent Claims					0	=	0

** or number previously paid, if greater, For reissues see below

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	78	202	39	Independent claims in excess of 3
104	260	204	130	Multiple dependent claim
109	78	209	39	Reissue independent claims over original patent
110	18	210	9	Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)18.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920	112	920	Requesting publication of SIR prior to Examiner action	
113	1,840	113	1,840	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	380	216	190	Extension for reply within second month	
117	870	217	435	Extension for reply within third month	
118	1,360	218	680	Extension for reply within fourth month	
128	1,850	228	925	Extension for reply within fifth month	
119	300	219	150	Notice of Appeal	
120	300	220	150	Filing a brief in support of an appeal	
121	260	221	130	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive unavoidably abandoned application	
141	1,210	241	605	Petition to revive unintentionally abandoned application	
142	1,210	242	605	Utility issue fee (or reissue)	
143	430	243	215	Design issue fee	
144	580	244	290	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	690	246	345	Filing a submission after final rejection (37 CFR 1.129(a))	
149	690	249	345	For each additional invention to be examined (37 CFR 1.129(b))	

Other fee (specify)

Other fee (specify)

* Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($)

SUBMITTED BY				Complete (if applicable)	
Typed or Printed Name	Jean C. Baker	Registration No. (Attorney/Agent)	35,433	Telephone	(414) 277-5709
Signature	Jean C. Baker			Date	September 15, 2000

ASSAYS FOR MODULATORS OF PROLYL-4-HYDROXYLASE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. provisional application Serial No. 60/154,267, filed September 16, 1999. Serial No. 60/154,267 is incorporated by reference as if fully set forth herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

–

BACKGROUND OF THE INVENTION

Prolyl-4-hydroxylases (P4H) are enzymes that modify collagen in a manner that stabilizes the conformation of collagen. The synthesis of hydroxyproline residues by P4H is a critical step in intracellular collagen processing.

Reduced P4H enzyme activity leads to unstable collagen and disease symptoms such as those seen in patients suffering from scurvy. Increased activity creates less pliable tissue and is associated with fibrotic diseases. P4H is recognized as an ideal target for the pharmacological control of collagen biosynthesis (Bickel, et al., Hepatology August:404-405, 1998).

BRIEF SUMMARY OF THE INVENTION

We have discovered an assay for modulators of P4H enzyme activity in the nematode *Caenorhabditis elegans*. Loss of one isoform of prolyl-4-

hydroxylase causes the nematode to be short and fat, a morphology termed "dumpy" or "dpy". (There are other nematode genes that can be mutated to the dpy phenotype, but there are methods known to one of skill in the art for determining which gene is responsible for the phenotype.) Loss of the second isoform of prolyl-4-hydroxylase while retaining the first isoform of prolyl-4-hydroxylase gives the nematode no apparent phenotype. Mutations in both prolyl-4-hydroxylase isoforms in the same animal result in embryonic lethality. The embryos develop to the pretzel stage and then retract into a mass of cells. These phenotypes provide an easy assay for detecting changes in prolyl-4-hydroxylase activity.

In another embodiment of the present invention, one would introduce the human version of P4H-gene into a P4H-modified nematode and, thus, complement the P4H mutation. One would then expose the test chimeric nematode to a test compound and determine whether the test compound interferes with the P4H activity by examining whether the chimeric nematode or its progeny develop a phenotype that can be attributed to modified P4H activity. We predict that the P4H-modified nematode, which has been exposed to the test compound, will have a phenotype similar to the *dpy-18* mutant or the *phy-1* mutant or the combined *dpy-18*; *phy-1* double mutant phenotype.

In another embodiment of the present invention, one would attempt to recover P4H activity, thus indicating that the test compound is a P4H activator. In that embodiment, one would introduce a test compound to a

P4H-modified nematode and examine the nematode and its progeny for either recovered P4H activity or a phenotype demonstrating wild-type P4H activity.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 diagrams four different putative prolyl 4-hydroxylase inhibitors.

Fig. 2A and Fig. 2B graphs percent lethality versus concentration of P4H inhibitors. Inhibitor I is depicted in Fig. 2A and Inhibitor II is depicted in Fig. 2B.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the present invention is a system designed to look for modifiers (inhibitors and activators) of prolyl 4-hydroxylase activity.

Inhibitors that specifically target human prolyl 4-hydroxylase alpha subunits (I or II) could be used to help people suffering from fibrotic diseases. Activators that specifically target the human prolyl 4-hydroxylase could be used to help treat diseases with Scurvy-like symptoms (underhydroxylated collagen or unstable collagens). Inhibitors or activators that specifically target any protein or molecule with prolyl 4-hydroxylase activity which can function in place of the *dpy-18* gene in the transgenic assay could be used as nematode or drosophila pesticides.

In a preferred embodiment, the assay would take place as follows:

Test nematodes will be exposed to a test compound to assay the effect of the compound on prolyl 4-hydroxylase activity. Suitable test nematodes used will include *dpy-18* animal rescued by the human alpha I subunit of prolyl 4-hydroxylase, *dpy-18* animal rescued by the human alpha II subunit of prolyl 4-hydroxylase, wild type *C. elegans*, *dpy-18* mutants, *phy-2* mutants and various *dpy-18;phy-1* mutant combinations. We have included some of these test nematodes to screen for inhibitors of nematode P4H which could potentially be used as pesticides. Combinations of mutant phenotypes could be used to look for specific gene inhibition and potentially specific gene activation. (The Examples below describe the isolation and characterization of the mutants. In general *dpy-18* is a deletion isolated specifically as a knock-out of the P4H gene on chromosome III and PHY-2 is a deletion mutant isolated specifically as a knock-out of the P4H gene on chromosome IV.)

In the methods of the present invention, one may wish to use particular test nematodes with modified P4H activities. Friedman, et al. (Proc. Natl. Acad. Sci. USA 97(9):4736-4741, 2000, incorporated by reference as if fully set forth herein) describes the creation of mutants useful for the present invention. Particularly, Friedman, et al., 2000 describes the creation of *dpy-18* and *phy-2* mutations. In general, we refer to these mutations as "P4H-gene modified nematodes." We refer to the P4H-gene modified nematodes that have been rescued with a human P4H gene as "test chimeric nematodes" or "test chimeric *C. elegans*."

In one embodiment, the test chimeric nematodes or wild-type nematodes will be exposed to test compounds such as chemicals, gene products, and natural products, by various different methods. Preferably, the nematodes will be placed in a solution containing the test compound and soaked for a period of time, or the test compound may be placed directly in the growth medium or on a slide, or introduced through a hole in the egg shell or introduced into the animal by injection into the germline. A suitable length of time would be determined experimentally based on the compound of interest and the age at which one would like to expose the worm.

In one embodiment, the test compound is part of a combinatorial chemical library.

If the test compound is an inhibitor of prolyl-4-hydroxylase activity, we expect the nematode's progeny to appear dpy or die, depending on whether the inhibitor is gene-specific or knocks out both prolyl-4-hydroxylase genes. For example, if the inhibitor is gene-specific to the DPY-18 protein, the nematode will appear dpy. If the inhibitor is non-specific and knocks out both P4H genes, the progeny of the tested animal will have a lethal phenotype.

In another embodiment, one would examine the nematodes for the P4H activity level (preferably the P4H:proline ratio). A reduced P4H activity would indicate that the compound is an inhibitor.

In another embodiment of the invention, one could compare the amount of inhibitor needed to affect wild-type, *dpy-18* or *psy-2* mutants. *Dpy-18* and *psy-2* mutations will be more sensitive to inhibitor.

Worms with a dpy phenotype appear to be shorter in length (approximately two/thirds wild-type) when viewed with a dissecting microscope. Worms with a lethal phenotype appear to be dead embryos when viewed with a dissecting microscope.

Activators of prolyl- 4-hydroxylase will rescue the *dpy-18* or *phy-1* phenotype. Potentially, *phy-1* or *dpy-18* nematodes could be exposed to the test compounds and any redundant expression could be activated to rescue the mutant phenotype.

The Examples below and Friedman, et al., 2000, describe how to create suitable mutants in *C. elegans*. Preferably, the nematode will be one of the genus *Caenorhabditis*, preferably *C. briggsae*. If one wished to use another nematode, such as *C. briggsae*, one of skill in the art would be able to create analogous mutants using the presented information.

EXAMPLES

Experimental Procedures

Worm strains

All wild-type *C. elegans* were from an N2 Bristol strain. Worms were cultured at 20°C under standard conditions unless otherwise noted (J.E. Sulston and J. Hodgkin, Methods. In The Nematode *Caenorhabditis elegans*, pp. 587-606, 1988). LG II:*unc-4(e120)* was used as a marker for transgenic assays. LG III:*dpy-18(ok162)* is a deletion mutation isolated specifically as a knock-out of the prolyl 4-hydroxylase on chromosome III. We found that *dpy-*

18 phenotype corresponds to the absence of prolyl 4-hydroxylase. 11 alleles (mutations in the *dpy-18* gene) are known--*dpy-18*: *e346, e364, e499, e1096, e1270, e1862, h662, s361, s1304, s1305, s1306.* LG IV:*unc-22(e66)* is a mutation that can be used to recognize chromosome IV, and *poh–1(ok-177)* is a deletion mutation isolated specifically as a knock-out of the prolyl 4-hydroxylase gene on chromosome IV.

hT2(I:III) is a rearrangement that contains a mutation in the *dpy-18* gene. Thus, *Ht2(I:III)* has a dpy phenotype and is not complemented by *dpy-18* mutations.

Description of Prolyl 4-hydroxylase Genes in *C. elegans*

Our searches using FASTA and BLAST with the human prolyl 4-hydroxylase sequence against the *C. elegans* genome revealed the presence of two *C. elegans* genes with homology to prolyl 4-hydroxylase. Y47D3B.10 is the transcript which corresponds to the prolyl 4-hydroxylase on LGIII (which we have determined to correspond to the *dpy-18* gene) and F35G2.4 is the transcript which corresponds to the prolyl 4-hydroxylase on LGIV. Phylogenetic analysis of the two genes compared with that of alpha I and alpha II of human, mouse, rat, chicken, drosophila and a virus prolyl 4-hydroxylase using the programs PILEUP of GCG and PAUP suggest that the two genes are more closely related to each other than to any other sequences.

Isolation of Deletion Mutants

To induce deletion mutations in the two different prolyl 4-hydroxylase genes in *C. elegans* we sent the following primers to Robert Barstead and Gary Moulder at the Oklahoma Medical Research Foundation. These researchers provide a service to the *C. elegans* community by isolating deletions in PCR screens of mutagenized populations. L4 hermaphrodites were treated with trimethylpsoralen and UV light as described (see http://snmc01.omrf.uokhsc.edu/revgen/RevGen.html and Dernburg, et al., Cell 94(3):387-398, 1998, for a protocol).

Offspring from mutagenized animals were cultured in groups of 500. After one generation genomic DNA was prepared from pools of worms, and nested primers were used in two successive rounds of PCR. The external primers for Y47D3B.10 (corresponding to *dpy-18*) were CACGACGAGGAAGAGCGACTG and TACGATTTCCAGTTCCCAAGC; the internal primers were GAAGAAGCTGTCGGAGGAGTA and ACGGCTAGTGGGTTGAATCTC. The expected product from amplification of wild-type genomic DNA is 3.2 kb. The external primers for F35G2.4 (corresponding to *poh–1*) were GCTCATGCAGATTTGTTCACT and GTCAGCAGGAAGGCAGTAAAC; the internal primers were GAGCAGAGAAGGATGTAACAA and ATAGTGCGCATTTCCGTTTCA. The expected product from amplification of wild-type genomic DNA is 2.8 kb.

Analysis of Hydroxylated Proline:Proline in Worm Cuticles

As a measure of prolyl 4-hydroxylase activity, the ratio of

4-hydroxyproline:proline was determined in the highly collagenous worm cuticle.

Isolation of Cuticles

To isolate cuticles, worms were bleached and embryos were collected and washed extensively in M9. Embryos were allowed to hatch overnight in M9 and then collected and washed and plated and allowed to grow to L4. L4 worms were collected and washed in M9 and frozen at –80°C. 2ml of packed worms were defrosted and washed with sonication buffer.

Cuticle isolation was performed as a modification of Edgar, et al., 1981. Nematodes were suspended in 3 ml of sonication buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride [PMSF], and given ten 20 second bursts of a Branson Sonifier 450 at 50% Duty Cycle and 5-7 output control. Cuticle pieces were collected by centrifugation for 4 minutes at 2000 x rpm in a Sorvall Super T21 and washed several times with 10 ml sonication buffer. Cuticles were then transferred to a 1.5 ml microfuge tube, suspended in 1 ml of ST buffer (1% SDS, 0.125 M Tris-HCl, pH 6.8) and heated for 2 minutes in a boiling water bath. The sample was then incubated for 6 hours, spun down for 60 seconds in an Eppendorf microcentrifuge, extracted again with ST buffer as described and left shaking overnight. The disulfide cross-linked proteins of the cuticle were solubilized by heating purified cuticles for 2 minutes in a boiling water bath in 0.5 ml of ST buffer with 5% β-mercaptoethanol (BME). The sample was rocked for 6 hours on a platform shaker and the solution was extracted and the sample was treated

for a second time and left to rock overnight. The insoluble cuticle material was washed several times with distilled water and speed vac dried. All protein samples were stored at –20°C.

Samples for amino acid analysis were hydrolyzed in 6N HCl/0.1% phenol at 110°C for 22 hours and assayed for the ratio of 4-hydroxyproline:proline at MIT's Biopolymer laboratory (Cambridge, MA).

Phenotypes

After receiving deletion mutants in the two prolyl 4-hydroxylase genes we analyzed the phenotypes of the individual mutants and the double mutants.

The fact that the dpy phenotype corresponds to the prolyl 4-hydroxylase on LGIII provides an easy method of assaying loss of function of this gene. If one knocks out *dpy-18*, one gets a short, fat, little worm, hence the name "dpy" for dumpy. The *phy-2* gene is wild type at 20°C but is more sensitive to inhibitor concentration than is the wild-type worm, thus allowing one to identify the specific knock-out of this gene.

The double mutant phenotype *dpy-18;phy-2* is an extremely embryonic lethal animals allowing us to look for inhibitors of both genes or all prolyl 4-hydroxylases.

RNAi:

Double-stranded RNA was produced using PCR-generated fragments of *phy-1* and *dpy-18* cDNA with T7 promoters linked to primers specific to said DNA. The RNA was then produced using the T7 MegaScript RNA kit

(Ambion). The RNA was injected at 5 mg/ml into N2 animals individually and in combination. The worms were grown at 15°C, 20°C and 25°C. RNA interference technology may be used to create the same knock-out phenotypes as those seen by the deletion mutations.

Proposed Isolation of the Human prolyl 4-hydroxylase Alpha I and Alpha II Subunit cDNAs.

Below we describe a proposed method of isolating human P4H gene. One of skill in the art would be aware of modifications and alternative methods that would be equally suitable.

The two full-length human prolyl 4-hydroxylase mRNAs have been described in Helaakoski, et al. 1994 (T. Helaakoski, et al., J. Biol. Chem. 269(45):27847-54, 1994) and Annunen, et al., 1997 (P. Annunen, et al., J. Biol. Chem. 272(28):17342-8, 1997.) respectively. Using the sequences described in the above mentioned papers Genebank ACCESSION # M24486, and M24487 corresponding to the two alpha I subunits and ACCESSION # U90441 corresponding to the alpha II subunit one could use the standard BLAST program and search the Genbank database for IMAGE consortium clones.

If one cannot obtain a full length clone from the IMAGE consortium one could use standard methods such as RT-PCR to create a full-length cDNA from human RNA or a human cDNA library.

Small Molecule Inhibition of Prolyl 4-Hydroxylase Activity.

Small molecules that inhibit protein function can be used to confirm and extend results from genetic experiments. We tested two known prolyl 4-hydroxylase inhibitors for their effects on *C. elegans*. Fig. 1 shows the structures of these inhibitors: 2,4-diethylpyridine dicarboxylate and dimethyloxalylglycine (Inhibitor I and Inhibitor II, respectively). Both inhibitors limit prolyl 4-hydroxylase activity in cells, where their esters are hydrolyzed to form competitors of α-ketoglutarate. We also tested Inhibitor III (which is similar in structure to Inhibitor II) and Inhibitor IV (which is similar in structure to Inhibitor I). Neither Inhibitor III nor Inhibitor II is known to limit prolyl 4-hydroxylase activity in cells.

We exposed adult hermaphrodites that were genotypically wild-type, *dpy-18(ok162)* or *phy-2(ok177)* to varying concentrations of inhibitors. The animal placed in inhibitor was apparently unaffected, but dramatic effects were observed among their progeny. Indeed, when exposed to a high level of Inhibitor I or II (2.7 μM and 1.3 μM, respectively), all progeny died, regardless of genotype (Fig 2A and 2B). The dead embryos arrested at the two-fold stage and then exploded; a phenotype reminiscent of the *dpy-18*; *phy-2* dead embryos. This suggests that exposure to the inhibitors results in a lowered prolyl 4-hydroxylase activity.

At a 10-fold lower concentration, the inhibitors affected *dpy-18(ok162)*, but not *phy-2(ok177)* progeny. To ask whether animals with a Dpy phenotype were unusually sensitive to inhibitor, we tested *dpy-10(e128)*, *dpy-11(e224)*,

dpy-13(e184), *dpy-17(e364)* and *dpy-20(e1282)* mutants for inhibitors effects. However, these other *dpy* mutants were comparable to wild-type animals in their response to both inhibitors. Therefore, the sensitivity of *dpy-18* mutants to inhibitors is not caused by its Dpy phenotype. In *dpy-18* mutants, the only prolyl 4-hydroxylase activity remaining is PHY-2, and conversely, in *phy-2* mutants, the only remaining activity is DPY-18. We suggest that the effect of the inhibitor on *dpy-18* mutants reflects inhibition of the remaining PHY-2, and vice versa. Because *dpy-18*, but not *phy-2*, progeny were affected by inhibitor at low concentration, we suggest that PHY-2 is either less abundant or more sensitive than DPY-18.

Both Inhibitor III (at ≤29 μM) and Inhibitor IV (≤3.2 mM) had no effect on the viability of *dpy-18* worms. (See Fig. 1 for structure of Inhibitors III and IV.) These two molecules had not been described previously as inhibitors of P4H.

CLAIMS

We claim:

1. A method for evaluating a test compound's ability to modulate prolyl-4-hydroxylase (P4H), comprising the steps of:

(a) introducing a test compound into a test chimeric nematode, a P4H-gene modified nematode, or a wild-type nematode, wherein the test chimeric nematode has a complemented prolyl-4-hydroxylase gene mutation, and

(b) observing the effect of the test compound on the prolyl 4-hydroxylase activity of the progeny of the test nematode, P4H-gene modified nematode or the wild-type nematode, wherein a dpy or embryonic lethal phenotype indicates prolyl-4-hydroxylase inhibition.

2. The method of claim 1, wherein the test compound is a chemical.

3. The method of claim 1, wherein the inhibitor is a protein or peptide.

4. The method of claim 1, wherein the introduction of the test compound involves placing the nematode in a solution containing the test compound.

5. The method of claim 1, wherein the test compound is introduced into a wild-type nematode and the observation of dpy or embryonic lethal phenotype indicates nematode prolyl 4-hydroxylase inhibition.

6. The method of claim 1, wherein the test compound is introduced into a P4H-gene modified nematode and the observation of a dpy or embryonic lethal phenotype indicates P4H inhibition.

7. The method of claim 1, wherein the introduction of a test compound is into a test chimeric nematode and the observation of dpy or embryonic lethal phenotype indicates non-native prolyl 4-hydroxylase inhibition.

8. The method of claim 1, wherein the test chimeric nematode is a *C. elegans* and is a *dpy-18* mutation.

9. The method of claim 1, wherein the observation of a dpy phenotype indicates that the test compound modulates the P4H gene found on chromosome III.

10. The method of claim 1, wherein the nematode is a member of the genus *Caenorhabditis*.

11. The method of claim 1 wherein the nematode is *C. elegans*.

12. A method for evaluating a test compound's ability to modulate prolyl 4-hydroxylase, comprising the step of:

(a) introducing a test compound into a nematode comprising a *dpy-18* or *poh-1* mutation phenotype, and

(b) observing the effect of the test compound on the prolyl-4-hydroxylase activity of the progeny of the test nematode, wherein the rescue of the dpy-18 or phy-1 phenotype indicates an increased level of prolyl-4-hydroxylase activity.

13. The method of claim 12 wherein the nematode is a member of the genus *Caenorhabditis*.

14. The method of claim 13 wherein the nematode is *C. elegans*.

15. The method of claim 1 wherein the test compound is part of a combinatorial chemical library.

16. The method of claim 12 wherein the test compound is part of a combinatorial library.

17. A method for evaluating a test compound's ability to modulate P4H, comprising the steps of:

(a) introducing a test compound into a test chimeric nematode, a P4H-gene modified nematode, or a wild-type nematode, wherein the test chimeric nematode has a complemented P4H gene mutation, and

(b) measuring the level of P4H activity of the progeny of the test nematodes, P4H gene modified nematode or wild-type nematode, wherein a lower P4H activity compared to untested control nematodes indicates that the test compound is an inhibitor of P4H.

18. The method of claim 17 wherein the measurement of P4H activity is via a ratio of P4H to proline.

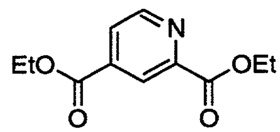
19. The method of claim 17 wherein the nematode is a member of the genus *Caenorhabditis*.

20. The method of claim 19 wherein the nematode is *C. elegans*.

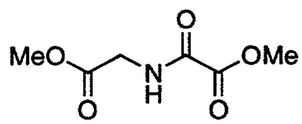
21. The method of claim 17 wherein the test compound is part of a combinatorial library.

ABSTRACT OF THE DISCLOSURE

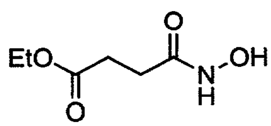
A method for evaluating a test compound's ability to modulate prolyl 4-hydroxylase is disclosed. In one embodiment, the method comprises the steps of introducing a test compound into a test chimeric, P4H-gene modified, or a wild-type nematode, wherein the test chimeric nematode has a complemented prolyl-4-hydroxylase gene mutation, and observing the effect of the test compound on the prolyl 4-hydroxylase activity of the progeny of the test chimeric, P4H-gene modified, or the wild-type nematode, wherein a dpy or embryonic lethal phenotype indicates prolyl-4-hydroxylase inhibition.



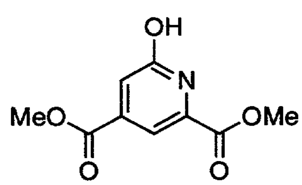
Inhibitor I



Inhibitor II



Inhibitor III



Inhibitor IV

FIG. 1

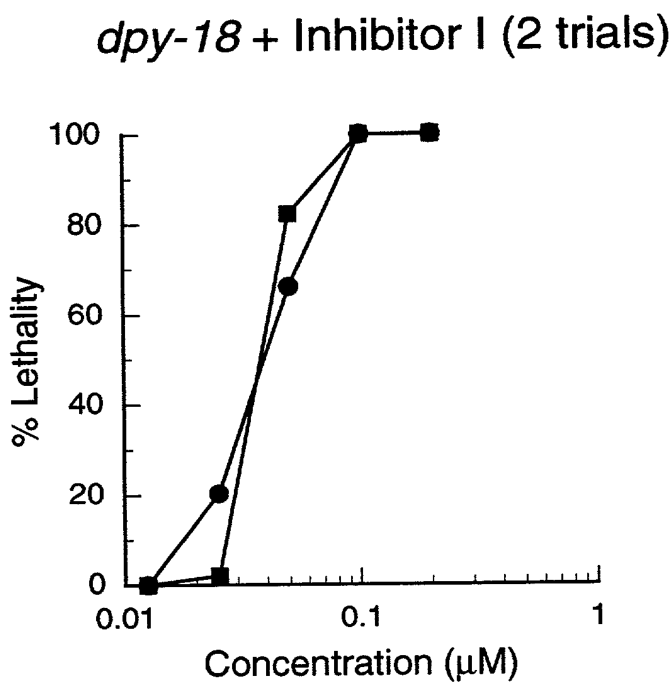

dpy-18 + Inhibitor I (2 trials)
100
80
60
40
20
0
% Lethality
0.01
0.1
1
Concentration (µM)

FIG. 2A

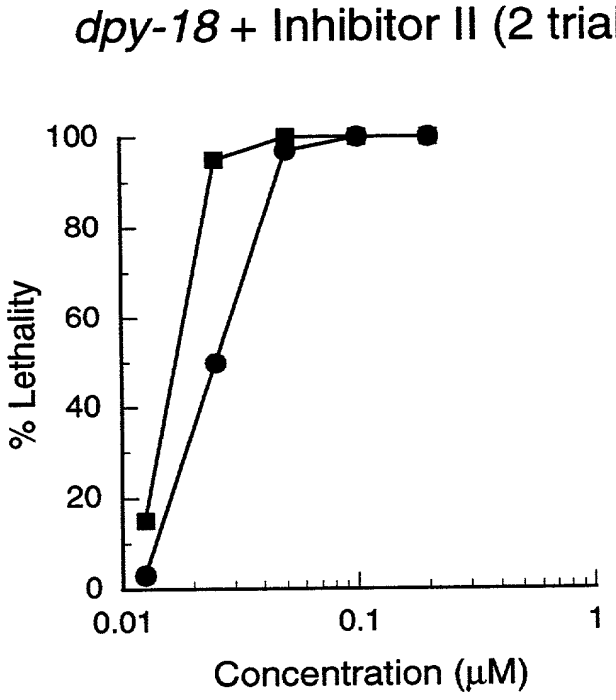


FIG. 2B

EXPRESS MAIL LABEL NO. EJ636887080US

PTO/SB/01 (6-95)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Please type a plus sign (+) inside this box [+]

0010/PTO Rev 6/95 — U.S. Department of Commerce Patent and Trademark Office

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

[X] Declaration Submitted with Initial Filing OR [ ] Declaration Submitted after Initial Filing

Attorney Docket Number	960296.96650
First Named Inventor	Judith E. Kimble
COMPLETE IF KNOWN	
Application Number	
Filing Date	September 15, 2000
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ASSAYS FOR MODULATORS OF PROLYL-4 HYDROXYLASE

(Title of the Invention)

the specification of which

[X] is attached hereto

OR

[ ] was filed on (MM/DD/YYYY) _____ as United States Application Number or PCT International Application Number _____ and was amended on (MM/DD/YYYY) _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1 56

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
n/a			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign applications numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	
60/154,267	September 16, 1999	[ ] Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

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DECLARATION

I hereby claim benefit under Title 35, United States Code §120 of any United States application(s), or §365(C) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or PCT international application in the manner provided in the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*
n/a			

[ ] Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and all continuation and divisional applications based thereon, and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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[ ] [X] Additional inventors are being named on supplemental sheet(s) attached hereto

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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet

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Post Office Address	

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Name of Additional Joint Inventor, if any | | | A petition has been filed for this unsigned inventor

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Inventor's Signature		Date	

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Post Office Address	
Post Office Address	

City		State		Zip		Country		Applicant Authority	

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